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The feasibility of the zebrafish embryo as a promising alternative for acute toxicity test using various fish species: A critical review

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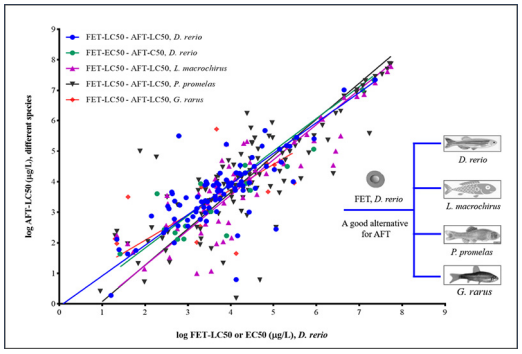
HIGHLIGHTS

- A positive relationship was exhibited between zebrafish FET and AFT.
- CTD models with review data validated the use of zebrafish FET for AFT prediction.
- Zebrafish FET EC50s well predicted acute toxicity for various fish species.
- Zebrafish FET can serve as an alternative for Chinese rare minnow AFT.

GRAPHICAL ABSTRACT



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ABSTRACT

With the European Union's restrictions on toxicity tests using vertebrates and the issue of the Organization for Economic Co-operation and Development test guideline 236, the fish embryo test (FET) has become a promising alternative to acute fish toxicity test (AFT), with zebrafish embryos being used the most. A large number of studies showed zebrafish FET correlated well with zebrafish AFT, yet its representativeness for other fish species is still under evaluation. In this review, toxicity data of zebrafish FET were summarized and compared with AFT using zebrafish and other test species in various countries, such as fathead minnow (*Pimephales promelas*), bluegill sunfish (*Lepomis macrochirus*), and Chinese rare minnow (*Gobiocypris rarus*). Previous findings of good relationship between zebrafish FET and AFT were confirmed, with a significant relationship between the median lethal and effect concentrations (LC50 and EC50) in FET and AFT LC50 for 87 chemicals (log ($LC50_{AFT}$) = 1.00 × log ($LC50_{FET}$) − 0.0829, $p < 0.0001$). Since embryo lethality generally occurs at chemical concentrations much higher than their environmentally relevant concentrations and involves in multiple modes of action, the EC50s gradually become the preferred FET parameters. Therefore, the use of FET EC50 for predicting fish toxicity was also evaluated. Interestingly, we found that the zebrafish FET toxicity data well predicted AFT of *G. rarus* that is native AFT model species in China, yet its corresponding FET is still immature. This highlights the zebrafish embryo test would serve as a good alternative for AFT across fish species.

1. Introduction

With the expansion of living quality requirements, the number of synthetic chemicals used in daily life is increasing at an unprecedented rate. According to the Global Chemicals Outlook, annual output of chemical industry exceeds five trillion US dollars nowadays, and chemical

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sales are expected to double from 2017 to 2030 (Johnson et al., 2020). The ever-growing use of chemicals resulted in a considerable amount of contaminants being discharged into the environment, posing potential risk to the ecosystem and humans (Brooks et al., 2020; Johnson et al., 2020). As a consequence, greater requirements for environmental safety of the bursting new chemicals captured more and more attention. More than 80% of synthetic chemicals, however, have limited or no environmental safety information, as a result of the high cost of animal toxicity tests (Hartung, 2009; Nagel, 2002).

At present, toxicity tests are generally classified into two types, i.e., *in vitro* tests using cells and tissues and *in vivo* animal tests. Although *in vitro* bioassays are high-throughput, animal toxicity tests provide more environmentally relevant toxicity information, which serves as a transition between a cell and human evaluation. Mammals, such as mice, rats, and rabbits are commonly used as experimental animals for animal toxicity tests, but some defects such as long life-cycle, slow reproduction rate, high cost, and complicated operation make them merely be good candidate methods in high tier risk evaluation (Hartung, 2009; Muthulakshmi et al., 2018; Nagel, 2002). Also, traditional chemical risk assessments cannot keep up with the frequently launched new chemicals. Long test duration, high cost, and animal ethics make it impossible to meet the needs of emerging chemical toxicity test (Judson et al., 2009). Therefore, it is essential to develop high-throughput and low-cost alternative toxicity testing methods (Zhang et al., 2018). Zebrafish embryo is considered as an alternative because of its short development cycle, transparency, easy acquisition, and simple experimental operation (Collins et al., 2008; Nagel, 2002). Some studies found good relationships between the acute toxicity results of fish embryos and adult fish for various contaminants while using the embryos saved experimental time and cost (Braunbeck et al., 2005; Lei et al., 2018; Nagel, 2002). Therefore, the German Wastewater Charging Act proposed the zebrafish fish embryo toxicity test (FET) to replace the fish acute toxicity test (AFT) (DIN, 2003), and a recent study also suggested to use FET to replace AFT as a universal wastewater evaluation model (Stelzer et al., 2018).

The use of FET is in accordance with the replacement, reduction, and refinement (3R) principles that have always been the goal and principle of mammalian toxicity testing (Russell and Burch, 1959). In the recent two decades, the 3R principles have been put up on stage again, and the corresponding measures have been taken. The implementation of Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) regulations in 2006 clearly stated that an alternative plan to promote non-animal testing was introduced, and testing on animals (vertebrates) began to be restricted in 2009 (Webster, 2005; European Commission, 2006). At the same time, there are also some specific regulations related to the 3R principles in individual European countries, such as Germany's requirement to use fish embryos only for wastewater testing (DIN, 2003; ISO, 2007) and the British Animal Protection Act (DEFRA, 2006). Due to the expected global demand for testing chemical management, the urgent call for alternative methods was also revealed by the promotion of Canada's Domestic Substances List (Canada Gazette, 1999), the REACH, and the ongoing Economic Cooperation and Development (OECD)/Environmental Protection Agency (EPA) high production volume (HPV) Challenge Program (OECD, 2004).

As fish acute toxicity data are generally required for chemical risk assessment (Bradbury et al., 2004), there is an increasing trend to develop the FET as an alternative for AFT (Embry et al., 2010). In 2010, based on the 3R principles, the United Nations (UN) Directive 2010/63/EU Act that strikes for the protection of animals regarding scientific purposes, claimed that the death of test fish should be avoided during toxicity tests and the fish should be achieved to a humanity's end with efforts (Herrmann et al., 2019). In 2013, the OECD adopted the test guideline 236 (OECD TG 236) with zebrafish embryos being used as the test subject. All these regulations and guidelines collectively illustrate that zebrafish FET will play a significant role in future animal alternative test (Braunbeck et al., 2015; OECD, 2013).

In company with the issue of OECD 236, the viability of FET for AFT was also assessed. Belanger et al. (2013) found a remarkable correlation for zebrafish FET to zebrafish AFT ($\log LC50_{FET} = (0.989 \times \log LC50_{AFT}) - 0.195$; $n = 72$, $r = 0.95$, $p < 0.001$). This study validated the OECD 236 test method, confirming the feasibility of using the zebrafish FET to predict the AFT data. On the other hand, Kluver et al. (2015) found that an increasing number of chemicals showed weak and/or no toxicity to zebrafish embryos, leading to deviations from the linear relationship. Even some chemicals are somehow toxic to zebrafish embryos, the chemicals present in the natural ecosystem at trace concentrations, their risk could be challenging to assess in water quality monitoring using the zebrafish FET median lethal concentrations (LC50). Alternatively, the use of the median effective concentration (EC50) values becomes more practical (Kluver et al., 2015; Lammer et al., 2009).

While the FET vs. AFT regression evaluation is usually performed for zebrafish, a variety of fish species are recommended as the test fish in different countries, as a result of the different local environment conditions (Lammer et al., 2009). Recent studies showed that in addition to zebrafish, the FET may also be a good AFT alternative for some other model fishes, such as *Pimephales promelas* and *Lepomis macrochirus* (Table S1 in the Supplementary Data). The evaluation is needed to be expanded when more test fish species are introduced.

Chinese rare minnow (*Gobiocypris rarus*) has been promoted to be used in the AFT following the OECD TG 203 protocol (OECD, 1992) as a native test species for the chemicals registered in China (The General Administration of Quality Supervision, 2013). As the share of Chinese market in the global chemical production and consumption is continuously increasing, it is expected that the requirement for fish toxicity data regarding *G. rarus* will also increase year by year, calling for the development of alternative test methods for this species (Brooks et al., 2020; Johnson et al., 2020; Liang and Zha, 2016). Although there were few investigations on this subject, no standard FET protocol using Chinese rare minnow has been developed yet. As such, the application of *G. rarus* FET for predicting fish toxicity is not feasible until the standardization of FET using *G. rarus*. Instead, the feasibility of using the mature zebrafish FET method as an alternative for *G. rarus* AFT is worthy of validation at the current stage.

The main goal of the present study is to assess the viability of zebrafish FET as the alternative for AFT using different fish species, including Chinese rare minnow, to obtain chemical toxicity information. To do so, the present study first verified if the zebrafish FET, including FET-$LC50_{FET}$ and $EC50_{FET}$, can act as a good alternative for the AFT test using the same species, then evaluated if the zebrafish FET would serve as a good alternative for other widely used model fish species, and at last, explored the accuracy of using zebrafish FET for predicting toxicity data of Chinese rare minnow, the recently proposed test species in China.

2. Methods

2.1. Data collection

Literature data collected in this study included two sets, FET and AFT. The AFT data regard four freshwater fishes, including zebrafish (*D. rerio*), fathead minnow (*P. promelas*), bluegill sunfish (*L. macrochirus*), and Chinese rare minnow (*G. rarus*), whereas only zebrafish FET data were included.

These data were collected 1) by searching several literature search engines and databases, such as Google Scholar (https://scholar.google.com/), CNKI (https://www.cnki.net/), PubMed, and Web of Science; 2) by using the USEPA as the core collection dataset, including the public USEPA ECOTOX database (http://cfpub.epa.gov/ECOTOX/), USEPA risk assessment tool (ASTER) database (http://www.epa.gov/Med/Prods_Pubs/aster.htm), and pesticide info database (http://www.pesticideinfo.org/

Index.html); and 3) from the references in the reviews published previously (Belanger et al., 2013; Lammer et al., 2009; Padilla et al., 2012; Selderslaghs et al., 2012).

After collection, all data were entered into an excel spreadsheet (Table S2) and statistically evaluated for the following parts, including chemical name (used to distinguish chemicals), Chemical Abstract Service (CAS) number (used to identify essential information of chemicals), the name of fish species (for screening zebrafish species), experimental details (for subsequent filtering process), the method for reporting chemical concentrations (for following filtering process), exposure duration (for following filtering process), and the reference (to confirm the source of the study as well as the accuracy and reliability of the study). Table S2 summarized the FET and AFT data information, and the physicochemical properties of the chemicals, including molecular weight, water solubility, and log K_{ow} values.

2.2. Data screening

The collected data covered the research of zebrafish FET and AFT in the past 60 years, among which the data for zebrafish consisted of 960 FET and 284 AFT studies. Notably, the LC50 values dominated toxicity data, accounting for 80.3% of 960 FET studies. For data quality assurance, we screened the zebrafish FET data according to the OECD TG 236 method and the promulgated FET regulations (the UBA Contract Number The 85-203-422) published by the German Federal Environmental Protection Agency in 2005 (Braunbeck et al., 2005; OECD, 2013).

The screened data were selected based on the following standards. 1) Embryo data with an exposure stage of 0–6 hours post-fertilization (hpf) were selected for subsequent analysis. For example, if there were multiple FET data with different exposure stages for the same compound, only the data with an exposure stage of 0–6 hpf were used for the analysis. 2) Exposure time of FET is usually 48–120 h and the 96-h data were used preferentially if different exposure time intervals were applied for the same chemical, secondly followed by the data of 120, 72, and 48 h. 3) Experimental methods included flow-through, renewal/semi-static renewal, and static non-renewal. According to the OECD TG 236, the renewal/semi-static renewal method (test solution should be renewed every 24 h) was generally recommended to ensure inter-assay imprecisions that were caused by concentration changes of the chemicals caused by degradation, volatilization, and other issues. Therefore, the renewal/semi-static renewal method was preferred and if there was no additional data, the static non-renewal method should be selected. 4) The measured concentration of the chemical can be used as a vital screening criterion. If multiple studies included the measured and non-measured chemical concentrations (nominal concentration), the measured concentration should be preferred. If no measured concentrations available, FET data with nominal concentrations were used.

Meanwhile, AFT data were also screened according to the OECD TG 203 (OECD, 1992), and the following screening criteria were considered for data selections. 1) Adult and juvenile fish should be selected for test. 2) Exposure time interval of the test should be 24–96 h. The 96-h LC50 value should be preferred if it accounted for over 90% of the overall data for a certain chemical, followed by 72-, 48- and 24-h LC50. 3) The renewal, flow-through, and the renewal/semi-static renewal methods should be preferentially selected, and then the static non-renewal method. 4) The measured chemical concentration should be firstly chosen, then the nominal concentration.

If multiple LC50 values were selected after screening the zebrafish FET and AFT data for the same chemical, these LC50 values were geometrically averaged, and the processed data were then used for the following analysis. In addition, if the LC50 value of a chemical was within a range and the ratio of its maximum to minimum was less than 3, the geometric mean of its maximum to minimum was included in the calculation, otherwise, it would be excluded (Belanger et al., 2013; Lammer et al., 2009). Moreover, in the case of mixture toxicity with multiple stressors, if a particular chemical was the main toxicity contributor, the data should be excluded.

A high log K_{ow} value was observed during data processing for the collected chemicals that had relatively low water solubility of less than 0.002 mg/L. As water solubility of the chemicals with large log K_{ow} was low, some substances were usually tested at concentrations exceeding their solubility limit and data obtained from these studies were questionable. Therefore, when conducting AFT-FET regression analysis, these data should be specifically evaluated (Belanger et al., 2013). Chemicals whose LC50 value/water solubility ratio greater than ten should be deleted if necessary. It can be seen from Fig. S1 that the LC50 value/water solubility ratio of most chemicals are between 0.1 and 10.

2.3. Data analyses

A chemical toxicity distribution (CTD) model was introduced to compare the sensitivity of FET and AFT using different species (Wang et al., 2014, 2018). To construct the CTD model, toxicity data are arranged in ascending order, and the following equation was used to calculate the percentile.

$$\text{Percentile} = i/(n+1) \times 100\% \quad (1)$$

where i is assigned to the level of LC50 (or EC50) values and n is the total number of chemicals. The percentile was converted to probit and the logarithm of LC50 is used to ordinate the CTD model (Wang et al., 2018). The ratio of LC50 corresponds to toxicity data of the same chemical to different fish species or zebrafish at different life stages. If a chemical has multiple corresponding LC50 values, several average values were used for analysis. If there are at least five known corresponding data, then the establishment of each CTD model can be applied to sensitivity analysis. Besides, the fitting of the CTD model can be generated by a log-normal model.

To compare the difference of CTD models between the FET and AFT, and whether the FET LC50 values could be good alternatives to the LC50 values of AFT, the following process is essential. First, threshold concentrations (TCs) at 1st, 5th, 10th, 50th, 90th, 95th, and 99th centiles were calculated using log-normal regression function from the corresponding values of FET and AFT's CTDs, respectively. Second, the ratio of TCs (TC_{FET}/TC_{AFT}) and its corresponding 95% confidence intervals (95% CI) were obtained by Monte Carlo simulation. The Crystal Ball software was used for the simulation. When the corresponding 95% CI of the TC ratio covered one, there was no significant difference, indicating the FET LC50 could serve as a good alternative to the AFT data.

In addition, since FET and AFT data were experimentally measured, the deviations existed. While in the conventional regression analysis, it was always considered as a hypothesis that measured responses would be affected by random variations. The statistical treatment was called the invariant variable model or the measurement error model (Fuller, 1987). If a conventional regression analysis was used, the regression's linear slope would be below estimation, and the responses obtained were used as the predicted variable proportional to the deviation level. In these data, the variability of FET data existed in the database from different toxicity values for the same chemical, and the variability of AFT data (e.g., LC50 values) was substantially the same. Orthogonal regression was used to fit the linear relationship between the two methods and was adjusted for the measured deviations (Jackson, 1991). In comparison to conventional regression, the amount of the orthogonal regression residual square of the vertical to the straight line was minimized, while the sum of standard regression residual sum of squares was minimized only in the vertical direction. The orthogonal regression can be well used in a forecasting analysis model if there are non-contradictions though it should be prudent in its application in variable problems (Carroll and Ruppert, 1996). Previous studies have provided detailed explorations of applying orthogonal regression to these types of datasets (Knobel et al., 2012; Lammer et al.,

2009; Rawlings et al., 2019; Selderslaghs et al., 2012). Graphpad prism 8.0 software was used to perform orthogonal regression analysis on the collected AFT and FET data.

For the two sets of data that were paired (AFT and FET), the following mode of action (MOA) allocation algorithms were used to provide a view of the compounds' MOA distribution in the database. These classifications included 1) distribution by OASIS (OASIS divides chemicals into different categories according to their toxic MOA. 2D structural information is used only to identify the MOA of chemicals. Based on theoretical and empiric knowledge the following seven hierarchically ordered MOA are distinguished (aldehydes; alpha, beta-unsaturated alcohols; phenols and anilines; esters; narcotic amines; basesurface narcotics)) using the OECD quantitative structure-activity relationship (QSAR) toolbox (Kienzler et al., 2017), 2) Verhaar classification (Verhaar et al., 1992), and 3) ECOSAR (Mayo-Bean et al., 2011). The purpose of the action method was to gain a better understanding of the diversity of substances in the database, not just the physicochemical descriptions of molecular weight, log K_{ow}, and solubility. This analysis enabled us to more comprehensively explain and analyze the toxicity of different compounds on fish and zebrafish embryos. The detailed information on the classification is shown in Tables S3–S5.

3. Results and discussion

3.1. Data analysis of zebrafish AFT and FET

The zebrafish AFT data were collected from 1954 to 2019 (Fig. S2). The booming investigations from 1954 to 1995 prompted the promulgation of the experimental protocols. The most widely used standard operating procedure nowadays is referred to as the OECD TG 203 guideline that was released in 1992. The same impact could be observed in zebrafish FET research between 2006 and 2013, which could tell from the issue of the REACH regulation in 2006, and the EU recommendation that using embryos as the alternative to fish in chemical toxicity tests in 2009. The OECD TG 236 guideline was then issued in 2013, recommending zebrafish embryos as the test subject. Therefore, we can also see from Fig. S2 that zebrafish FET studies surged from 2006 to 2013. From the perspective of both tests, the total number of zebrafish FET studies was much greater than that of zebrafish AFT and the different costs for conducting the tests well explained. Overall, the zebrafish FET test has already become the preferred method for chemical toxicity testing.

A total of 427 chemicals were collected for comparing FET and AFT using zebrafish. The majority of the chemicals had molecular weights in a range of 100 to 400, with few tests were conducted for macromolecular polymers. The log K_{ow} values of the chemicals cover a wide range from −5.83 to 7.54 and most were between −1 and 6. However, approximately a quarter of the test chemicals cannot find the log K_{ow} values, most of which are metals or inorganic chemicals. The water solubility of the chemicals varied greatly, ranging from 0.002 mg/L to 2170 g/L, and about a quarter of them cannot find solubility information. Regardless of the molecular weight, log K_{ow} or water solubility, the collected information covers a wide range (Fig. S3), suggesting that the massive dataset would be of great use in the comprehensive analysis.

After checking data quality, zebrafish AFT and FET data for 117 and 347 chemicals, respectively, were summarized. Most of the AFT data were collected from tests conducted with an exposure time of 96 h, accounting for over 95% of all studies, while the FET data were mainly from the research after 2006, with most had exposure time intervals of 96 and 48 h (42.5% and 39.4%, respectively). The uniformity of exposure time showed that the promulgation of OECD TG 236 guideline had a significant impact on chemical toxicity tests, and it also indicated that embryos as alternatives for AFT is already in progress.

3.1.1. CTD analysis

The collected zebrafish AFT and FET data were paired and screened based on the OECD TG 203 and 236, respectively. After screening, a total of 87 chemicals with paired AFT and FET data were used for the construction of CTD models (Fig. 1). As shown in Fig. 1, the constructed CTD models for FET and AFT data had almost overlapped data point distributions, and the slopes of two curves were almost the same. The linear fitting equations of CTD models for AFT and FET were $Y = 0.755\,X + 2.28$ ($r^2 = 0.939$, $n = 87$) and $Y = 0.750\,X + 2.240$ ($r^2 = 0.925$, $n = 87$), respectively. According to the equations, the 5th threshold concentrations (TC5) for FET and AFT were calculated, and their corresponding 95% CIs were also simulated using the Monte Carlo modelling. The TC5 (95% CI) values were 27.0 (19.4, 36.5) and 31.2 (21.4, 43.7) μg/L for AFT and FET, respectively, and the 95% CI of the two TC5 values were overlapped, which was inconsistent with the observation of similar data distribution in Fig. 1. The ratio of $TC5_{AFT}$ to $TC5_{FET}$ (95% CI) was also computed to be 0.864 (0.488, 1.54), and its 95% CI covered 1. Collectively, CTD models corresponding to AFT and FET had no significant difference and function mutually.

To better understanding the impact of MOA of individual chemicals on the feasibility of using FET to replace zebrafish AFT, the paired 87 chemicals were further classified into different MoA groups (Tables S3–S5). If only Verhaar classification was used, more than 40% of the compounds could not be classified, yet the percentage dropped to 8% when the rest were further classified by OASIS classification. In addition, over 73% of compounds can be classified when using USEPA's ECOSAR for classification (Tables S3–S5). The chemicals that were difficult to be classified were mainly salts and some surfactants. There were six categories of compounds based on the OASIS classification of MOA (Table S4). Approximately 15% belong to basesurface narcotics, 20% belong to phenols and anilines, and more than 50% belong to reactive unspecified. Similar to the analysis for all compounds, the CTD curves based on individual MOAs, regardless of basesurface narcotics, phenols and anilines, and reactive unspecified (Fig. S4 and Table S6), had overlapped 95% CI of the TC5s for FET and AFT, suggesting that their corresponding FET and AFT models were not significantly different. Overall, the CTD analysis validated the applicability of zebrafish FET as a replacement of zebrafish AFT that were restricted to perform in many countries. As the FET could be served as a good alternative for zebrafish AFT, regardless of chemical MOAs, it would play an important role in chemical risk assessment and the establishment of freshwater quality standard thresholds.

3.1.2. Orthogonal regression analysis

In addition to CTD analysis, zebrafish FET and AFT data for the 87 chemicals were also undergone an orthogonal regression analysis. Compared with a previous study by Belanger et al. (2013), the number of chemicals used for orthogonal regression analysis between the

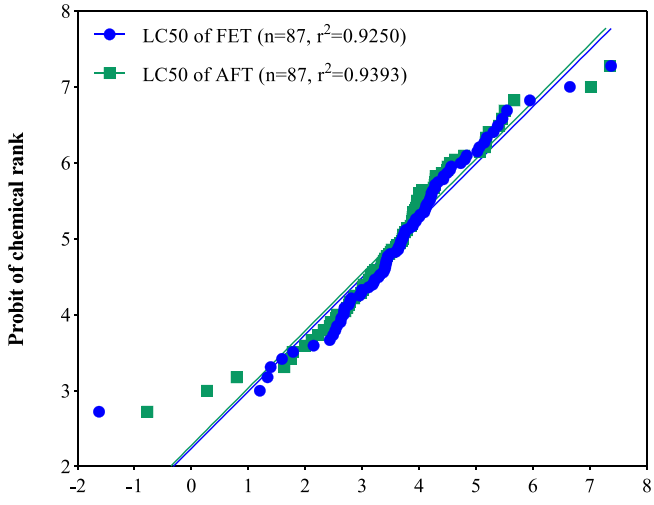

Fig. 1. Chemical toxicity distribution (CTD) models of zebrafish fish embryo test (FET) and acute fish toxicity test (AFT).

zebrafish and their embryos in the present study doubled (from 44 to 87). With more data in the analysis, the present study found that the regression slope for the LC50 values of zebrafish FET and AFT approaching 1, with a p value less than 0.0001 and an intercept close to 0 (Fig. 2A).

When only the tests with an exposure time of 96 h were considered, the number of available chemicals reduced to 56 (Fig. 2B). While the regression curve of AFT vs. FET slightly changed, the slope and intercept were still close to 1 and 0, respectively, showing that the zebrafish FET following OECD TG 236 was feasible to be a great replacement for zebrafish AFT (OECD TG 203). As shown in Fig. S5, based on the MOA classification of OASIS, orthogonal regression analysis was also individually performed on the three compound categories, i.e., basesurface narcotics, phenols and anilines, and reactive unspecified. The slopes and intercepts of the curves for different categories of compounds were close to 1 and 0, respectively.

Although most data were close to the 1:1 line, a small portion of chemicals had substantially higher LC50 values for zebrafish FET than their AFT LC50 values (Fig. 2). Kluver et al. (2015) reported considerably different sensitivity of fish and embryos to neurotoxic chemicals as these compounds generally had little impact on embryos in which the specific action sites for neurotoxicity were insufficiently developed. Thus, the application of LC50 of FET to predict the AFT LC50 would introduce high errors for this type of chemicals. In some cases, no lethality could be noted in the FET even chemical concentrations in water were much higher than their solubility. Instead, their easy-to-observe embryo life stage and sublethal effects of external stimuli during the development are more sensitive and observable than fish (DIN, 2003). The sublethal effects in zebrafish FET mainly included pericardial effusion, body curvature, yolk enlargement, short body length, and light pigmentation and can be observed under the optical microscope.

The promulgation of OECD TG 236 further expanded the application of zebrafish FET, not only towards individual chemicals, but also environmental mixtures, such as wastewater toxicity assessment (Braunbeck et al., 2015; Busquet et al., 2014; Scholz et al., 2016). Different from the toxicity tests using dosing chemicals in the laboratory, the composition and concentrations of chemicals in field-collected samples were unknown. Moreover, the toxicity of mixture samples was also complicated by transformation products, synergistic or antagonistic effects, and bioavailability of the compounds (Stewart and Carter, 2009). Therefore, it is reasonable that the test method designed for simple mixture exposure (OECD, 2013) may fail to gain effective toxicity evaluation for complex mixtures (Stelzer et al., 2018). Taking into account the uncertainty of chemical composition in the field and the possible low concentrations of key pollutants in surface water, it is strongly recommended to use the more sensitive toxicity testing methods in risk assessment (Hamilton et al., 2016; Schwarzenbach et al., 2006; Sobanska et al., 2018). Therefore, the evaluation of sublethal endpoints rather than lethality in zebrafish FET would provide more information in assessing the risk of field samples.

To promote the use of sublethal endpoints in zebrafish FET, the FET EC50 values were also collected and compared with the AFT data. A total of 26 chemicals were used in the regression analysis. Similar to the LC50 values, the slope of the regression curve between the zebrafish FET EC50 values and AFT LC50 was close to 1, and the intercept was close to 0 ($p < 0.0001$) (Fig. 2C). A linear regression between FET EC50 and AFT LC50 was also noted by Lammer et al. (2009). The establishment of the relationship between FET EC50 and AFT toxicity would help to predict fish toxicity for the chemicals that had relatively low lethality to embryos and field samples with multiple stressors.

3.2. Relationship of zebrafish FET with AFT of fathead minnow and bluegill sunfish

While zebrafish has been extensively used as model test species for AFT, other fish species were also widely used in the tests. Due to different natural environments, complex geographic factors, and a wide variety of fish distribution in the world, it is recommended to include native species for fish toxicity testing in addition to zebrafish, such as fathead minnow (*P. promelas*) and bluegill sunfish (*L. macrochirus*) (Lammer et al., 2009). The promulgation of OECD TG 236 means that fish embryos have become an indispensable alternative to AFT using various fish species. Previous studies have found that almost all FET data were using zebrafish embryos, accounting for more than 90% of the entire fish embryo toxicity data (Lammer et al., 2009; Rawlings et al., 2019). As discussed above, zebrafish FET well predicts zebrafish toxicity, however, it is still unclear if zebrafish FET would also serve as a good alternative to AFT using other fish species. Therefore, the relationships between zebrafish FET data with AFT using fathead minnow and bluegill sunfish were also explored in the present study. After checking data quality, the LC50 values of zebrafish FET and AFT of the two fishes were compared. The regression curves were $\log LC50_{AFT\text{-}P.\ promelas} = 1.19\ (1.02, 1.37) \times \log LC50_{FET\text{-}D.\ rerio} - 1.12$ ($p < 0.0001$, n = 115) and $\log LC50_{AFT\text{-}L.\ macrochirus} = 1.15\ (0.941, 1.36) \times \log LC50_{FET\text{-}D.\ rerio} - 1.04$ ($p < 0.0001$, n = 62) for fathead minnow and bluegill sunfish, respectively (Fig. S6). Both slopes were close to 1 and the p value was less than 0.0001, indicating that zebrafish FET could well predict the LC50 values in AFT using the two fishes. Different from the comparison within zebrafish species, the intercepts of the two curves deviated from 0, implying different toxicity sensitivity across species. This was consistent with a study conducted by Rawlings et al. (2019). Therefore, zebrafish FET conducted under OECD TG 236 should be promoted, not only the alternative between a single species, but also cross-species substitution seems to be a possibility.

3.3. Relationship between zebrafish FET and Chinese rare minnow AFT

In 2013, when the OECD TG 236 was issued, a standard AFT guideline using Chinese rare minnow was also promulgated in China (GB/T 29763–2013), officially recommending the Chinese rare minnow as

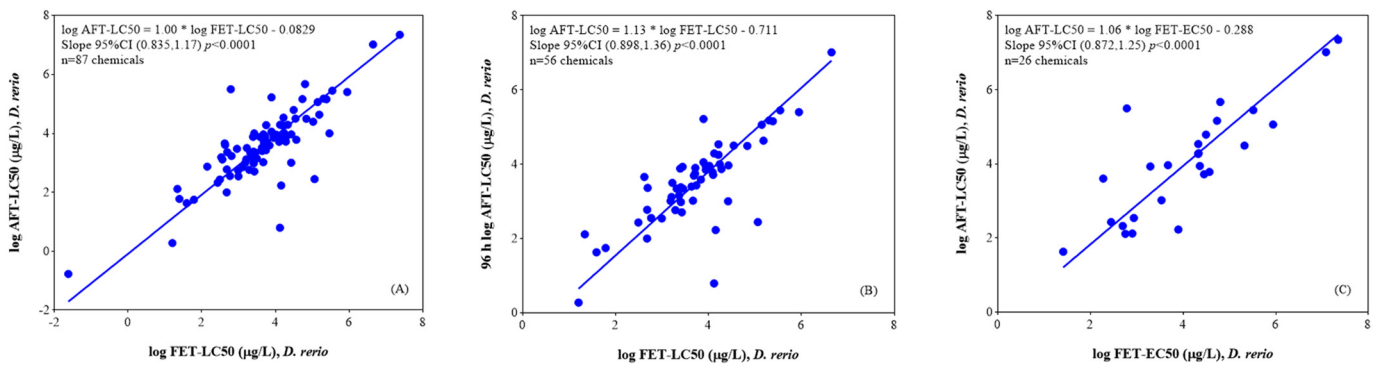

Fig. 2. Relationship between the zebrafish acute fish toxicity test (AFT) and fish embryo test (FET). (A), AFT median lethal concentrations (LC50) vs. FET LC50 for all tests, (B) AFT LC50 vs. FET LC50 for the 96-h tests, and (C), AFT LC50 vs. FET median effect concentrations (EC50).

the Chinese native model fish (General Administration of Quality Supervision, 2013). The issue of this guideline also means that the Chinese rare minnow will play an important role in the future chemical toxicity risk assessment or aquatic risk evaluation in China, therefore, the requirement of toxicity data for this species is increasing. On the other hand, with the continuous escalation of animal ethical issues and the promotion of 3R principles, to use embryos as an alternative of fish in toxicity testing is also inevitable in the future. It became a paradox for the ever-growing requirements for toxicity data with Chinese rare minnow and the restrictions of using fish in toxicity testing.

To find appropriate alternative test methods is the solution. As the use of Chinese rare minnow in AFT has just been issued in recent years, the FET using this species is still in its infancy and little information is available. In contrast to the mature zebrafish FET method, there is no uniform test protocol for Chinese rare minnow embryos up to now. Thus, it is essential to check the feasibility of using zebrafish FET as an alternative for AFT with Chinese rare minnow. At present, barely any research revealed the relationship between toxicity data with zebrafish and Chinese rare minnow. Hence, the present review collected the available literature AFT data using the Chinese rare minnow, and established a link between the LC50 values of Chinese rare minnow AFT and the LC50s of zebrafish FET (Fig. 3). This suggested that the toxicity to Chinese rare minnow could be predicted from zebrafish FET data, and thus it could be an important way to fill the data gap for Chinese rare minnow.

4. Conclusions

In the present review, the viability of using zebrafish FET data to predict AFT information for zebrafish and other commonly used test species (fathead minnow, bluegill sunfish, and Chinese rare minnow) was validated. With the issue of the standard test guidelines (e.g., OECD TG 236), data quality of zebrafish FET was more reliable, providing a cost- and labor-efficient way to gain fish toxicity information. This is of great importance for chemical management and water quality monitoring in a world with a continuously increasing number of chemicals. The relationship between the LC50 for zebrafish FET and Chinese rare minnow AFT was first built in the present study, providing a solution for the growing requirement of Chinese rare minnow toxicity data when chemical production and consumption have gradually shifted from the U.S. and Europe to Asia (Leung et al., 2020). In addition to the widely used zebrafish FET LC50, the EC50 values are also important indicators for assessing fish toxicity, particularly for chemicals whose LC50 are difficult to measure in zebrafish FET due to low lethality and/or low water solubility, as well as complex mixtures.

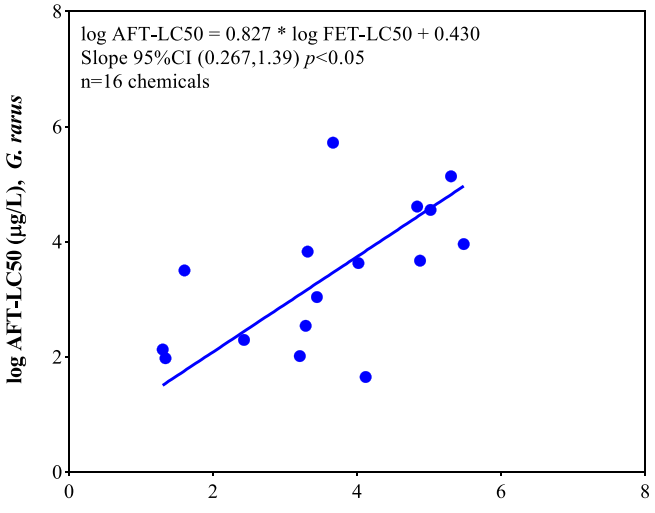

Fig. 3. Relationship of the median lethal concentration (LC50) values between the zebrafish embryo test (FET) and acute fish toxicity test (AFT) with Chinese rare minnow.

The criteria for screening zebrafish FET data were mainly based on the OECD TG 236, but some data in the studies that were conducted before the issue of the guideline can also be used after stringent checks, such as embryo selection time of 0–6 h. Before the promulgation of the OECD TG 236, numerous FET tests used embryos at 6 hpf, whether these data could meet the OECD TG 236 requirement needs further proof.

In this review, the LC50 of some FET is 10 times or 100 times lower than that of the AFT. However, limited research on these compounds is available currently, and the specific MOA is unclear. In contrast, for some compounds, the LC50 of the FET is 10 times or 100 times greater than the AFT (Knobel et al., 2012). Weak metabolism or low absorption affinity of zebrafish embryos may cause this. The lower LC50 value of FET, on the other hand, may be associated with high absorption affinity of these compounds in zebrafish embryos. The disparity between the LC50 of the zebrafish FET and the AFT, according to Knobel et al. (2012), may be caused by a large difference between the measured and nominal concentrations. Furthermore, certain compounds may not be appropriate for testing the association between FET and AFT if the LC50 value of FET is only used to research its correlation with the LC50 value of AFT. For example, when analyzing the relationship between zebrafish FET and AFT, it was found that although some compounds had low to no toxicity in FET, they showed hundreds or even thousands of times higher toxicity in AFT compared with their toxicity in FET. For example, the FET LC50 value of allyl alcohol is 794 times the LC50 value of the zebrafish AFT. Also, we found similar sensitivity in zebrafish FET and the AFT of fathead minnow (*P. promelas*) and bluegill sunfish (*L. macrochirus*), such as dieldrin and diquat dibromide. The correlation of the fitted prediction equation would decrease if these compounds are used in the correlation between zebrafish FET and different fish AFT. Actually, there is a strong association between zebrafish FET and AFT for the majority of compounds (85%), although the data quality check of the FET EC50 still needs to be improved. The issue of the standard test guidelines, such as OECD TG 236, would aid in the promotion of FET by improving the quality of toxicity data (LC50 and EC50), resulting in a more favorable guarantee for chemical risk management. Moreover, the universal use of zebrafish FET as a replacement for AFT for a variety of fish species, particularly the fish with limited toxicity data (e.g., Chinese rare minnow) also showed the advantage of promoting zebrafish FET in aquatic risk assessment.

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CRediT authorship contribution statement

Tenghui Su: Investigation, Formal analysis, Writing – original draft. **Deru Lian:** Writing – review & editing. **Yunfei Bai:** Investigation. **Yolina Yu Lin Wang:** Investigation. **Dainan Zhang:** Funding acquisition, Formal analysis, Writing – review & editing. **Zhen Wang:** Funding acquisition, Formal analysis, Writing – review & editing. **Jing You:** Conceptualization, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Belanger, S.E., Rawlings, J.M., Carr, G.J., 2013. Use of fish embryo toxicity tests for the prediction of acute fish toxicity to chemicals. Environ. Toxicol. Chem. 32, 1768–1783.

Bradbury, S.P., Feijtel, T.C., Leeuwen, C.J.V., 2004. Peer reviewed: meeting the scientific needs of ecological risk assessment in a regulatory context. Environ. Sci. Technol. 38, 463A–470A.

Braunbeck, T., Boettcher, M., Hollert, H., Kosmehl, T., Lammer, E., Leist, E., Rudolf, M., Seitz, N., 2005. Towards an alternative for the acute fish LC50 test in chemical assessment: the fish embryo toxicity test goes multi-species—an update. Altex. 22, 87–102.

Braunbeck, T., Kais, B., Lammer, E., Otte, J., Schneider, K., Stengel, D., Strecker, R., 2015. The fish embryo test (FET): origin, applications, and future. Environ. Sci. Pollut. Res. Int. 22, 16247–16261.

Brooks, B.W., Sabo-Attwood, T., Choi, K., Kim, S., Kostal, J., LaLone, C.A., Langan, L.M., Margiotta-Casaluci, L., You, J., Zhang, X.W., 2020. Toxicology advances for 21st century chemical pollution. One Earth. 2, 312–316.

Busquet, F., Strecker, R., Rawlings, J.M., Belanger, S.E., Braunbeck, T., Carr, G.J., Cenijn, P., Fochtman, P., Gourmelon, A., Hubler, N., Kleensang, A., Knobel, M., Kussatz, C., Legler, J., Lillicrap, A., Martinez-Jeronimo, F., Polleichtner, C., Rzodeczko, H., Salinas, E., Schneider, K.E., Scholz, S., van den Brandhof, E.J., van der Ven, L.T., Walter-Rohde, S., Weigt, S., Witters, H., Halder, M., 2014. OECD validation study to assess intra- and inter-laboratory reproducibility of the zebrafish embryo toxicity test for acute aquatic toxicity testing. Regul. Toxicol. Pharmacol. 69, 496–511.

Canada Gazette, 1999. Canadian Environmental Protection Act. Canada Gazette [accessed 19 December 2006]. 22, 3 Part III.

Carroll, R.J., Ruppert, D., 1996. The use and misuse of orthogonal regression in linear errors-in-variables models. Am. Stat. 50, 1–6.

Collins, F.S., Gray, G.M., Bucher, J.R., 2008. Toxicology. Transforming environmental health protection. Science. 319, 906–907.

DEFRA (UK Department for Environment, Food and Rural Affairs), 2006. Animal Welfare Act. Chapter 45. Department for Environmental Food, and Rural Affairs, London, UK.

DIN, 2003. German standard methods for the examination of water, waste water and sludge – subanimal testing (group T) – part 6: toxicity to fish. Determination of the Non-acute-poisonous Effect of Waste Water to Fish Eggs by Dilution Limits (T 6). DIN 38415-6.

Embry, M.R., Belanger, S.E., Braunbeck, T.A., Galay-Burgos, M., Halder, M., Hinton, D.E., Leonard, M.A., Lillicrap, A., Norberg-King, T., Whale, G., 2010. The fish embryo toxicity test as an animal alternative method in hazard and risk assessment and scientific research. Aquat. Toxicol. 97, 79–87.

European Commission, 2006. Regulation 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. Off. J. Eur. Union L 136, 3–280.

Fuller, W., 1987. Measurement Error Models. John Wiley & Sons, New York, NY, USA.

General Administration of Quality Supervision, Inspection and Quarantine of the People' s Republic of China, Standardization Administration of the People's Republic of China, 2013. Chemicals-Rare Minnow (*Gobiocypris rarus*) Acute Toxicity Test (GB/T 29763-2013). Standards Press of China, Beijing, China.

Hamilton, P.B., Cowx, I.G., Oleksiak, M.F., Griffiths, A.M., Grahn, M., Stevens, J.R., Carvalho, G.R., Nicol, E., Tyler, C.R., 2016. Population-level consequences for wild fish exposed to sublethal concentrations of chemicals — a critical review. Fish Fish. 17, 545–566.

Hartung, T., 2009. Toxicology for the twenty-first century. Nature. 460, 208–212.

Herrmann, K., Pistollato, F., Stephens, M.L., 2019. Beyond the 3Rs: Expanding the Use of Human-relevant Replacement Methods in Biomedical Research.

ISO, 2007. Water Quality-determination of the Acute Toxicity of Waste Water to Zebrafish Eggs (*Danio rerio*). 15088. International Standards Organization, ISO.

Jackson, J.E., 1991. A User's Guide to Principal Components. John Wiley & Sons, New York, NY, USA.

Johnson, A.C., Jin, X.W., Nakada, N., Sumpter, J.P., 2020. Learning from the past and considering the future of chemicals in the environment. Science. 367, 384–387.

Judson, R., Richard, A., Dix, D.J., Houck, K., Martin, M., Kavlock, R., Dellarco, V., Henry, T., Holderman, T., Sayre, P., Tan, S., Carpenter, T., Smith, E., 2009. The toxicity data landscape for environmental chemicals. Environ. Health Perspect. 117, 685–695.

Kienzler, A., Barron, M.G., Belanger, S.E., Beasley, A., Embry, M.R., 2017. Mode of Action (MOA) assignment classifications for ecotoxicology: an evaluation of approaches. Environ. Sci. Technol. 51, 10203–10211.

Kluver, N., Konig, M., Ortmann, J., Massei, R., Paschke, A., Kuhne, R., Scholz, S., 2015. Fish embryo toxicity test: identification of compounds with weak toxicity and analysis of behavioral effects to improve prediction of acute toxicity for neurotoxic compounds. Environ. Sci. Technol. 49, 7002–7011.

Knobel, M., Busser, F.J., Rico-Rico, A., Kramer, N.I., Hermens, J.L., Hafner, C., Tanneberger, K., Schirmer, K., Scholz, S., 2012. Predicting adult fish acute lethality with the zebrafish embryo: relevance of test duration, endpoints, compound properties, and exposure concentration analysis. Environ. Sci. Technol. 46, 9690–9700.

Lammer, E., Carr, G.J., Wendler, K., Rawlings, J.M., Belanger, S.E., Braunbeck, T., 2009. Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test? Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 149, 196–209.

Lei, K., Zhu, Y., Chen, W., Pan, H.Y., Guo, B.B., Zhang, X., Cao, Y.X., Sweetman, A.J., Lin, C.Y., 2018. The occurrence of home and personal care products in the Haihe River catchment and estimation of human exposure. Sci. Total Environ. 643, 63–72.

Leung, K.M.Y., Yeung, K.Y.W., You, J., Choi, K., Zhang, X., Smith, R., Zhou, G.J., Yung, M.M.N., Arias-Barreiro, C., An, Y., Burket, S.R., Dwyer, R., Goodkin, N., Hii, W.S., Hoang, T., Humphrey, C., Iwai, C.B., Jeong, S.-W., Juhel, G., Karami, A., Kyriazi-Huber, K., Lee, K.C., Lin, B., Lu, B., Martin, P., Nillos, M.G., Oginawati, K., Kathnayake, V., Risjani, Y., Shoeb, M., Tan, C.H., Tsuchiya, M.C., Ankley, G.T., Boxall, A.B.A., Rudd, M.A., Brooks, B.W., 2020. Towards sustainable environmental quality: priority research questions for Asia. Environ. Toxicol. Chem. 39, 1485–1505.

Liang, X.F., Zha, J.M., 2016. Toxicogenomic applications of Chinese rare minnow (*Gobiocypris rarus*) in aquatic toxicology. Comp. Biochem. Physiol. D. Genom. Proteom. 19, 174–180.

Mayo-Bean, K., Nabholz, J.V., Clements, R., Zeeman, M., Henry, T., Rodier, D., Moran, K., Meylan, B., Ranslow, P., 2011. ECOSAR Class Program for Estimating Toxicity of Industrial Chemicals to Aquatic Organisms Using the ECOSAR (Ecological Structure Activity Relationship) Class Program. MSWindows Ver 1.1. 22. Office of Pollution Prevention and Toxics, US Environmental Protection Agency, Washington, DC, pp. 545–559.

Muthulakshmi, S., Maharajan, K., Habibi, H.R., Kadirvelu, K., Venkataramana, M., 2018. Zearalenone induced embryo and neurotoxicity in zebrafish model (*Danio rerio*): role of oxidative stress revealed by a multi biomarker study. Chemosphere. 198, 111–121.

Nagel, R., 2002. DarT: the embryo test with the zebrafish *Danio rerio*—a general model in ecotoxicology and toxicology. Altex. 19, 38–48.

OECD, 1992. OECD Guideline for Testing of Chemicals Fish Acute Toxicity Test. OECD, Paris, France.

OECD, 2004. Manual for Investigation of HPV Chemicals. Organization for Economic Co-operation and Development, Paris, France.

OECD, 2013. OECD Guidelines for the Testing of Chemicals Section 2: Effects on Biotic Systems Test No 236: Fish Embryo Acute Toxicity (FET) Test. Organization for Economic Cooperation and Development, Paris, France.

Padilla, S., Corum, D., Padnos, B., Hunter, D.L., Beam, A., Houck, K.A., Sipes, N., Kleinstreuer, N., Knudsen, T., Dix, D.J., Reif, D.M., 2012. Zebrafish developmental screening of the ToxCast Phase I chemical library. Reprod. Toxicol. 33, 174–187.

Rawlings, J.M., Belanger, S.E., Connors, K.A., Carr, G.J., 2019. Fish embryo tests and acute fish toxicity tests are interchangeable in the application of the threshold approach. Environ. Toxicol. Chem. 38, 671–681.

Russell, W.M.S., Burch, R.L., 1959. The Principles of Humane Experimental Technique. Methuen.

Scholz, S., Kluver, N., Kühne, R., 2016. Analysis of the Relevance and Adequateness of Using Fish Embryo Acute Toxicity (FET) Test Guidance (OECD 236) to Fulfill the Information Requirements and Addressing Concerns under REACH. Report ECHA-UFZ (contract ECHA/2014/341). European Chemicals Agency, Helsinki, Finland.

Schwarzenbach, R.P., Escher, B.I., Fenner, K., Hofstetter, T.B., Johnson, C.A., von Gunten, U., Wehrli, B., 2006. The challenge of micropollutants in aquatic systems. Science. 313, 1072–1077.

Selderslaghs, I.W., Blust, R., Witters, H.E., 2012. Feasibility study of the zebrafish assay as an alternative method to screen for developmental toxicity and embryotoxicity using a training set of 27 compounds. Reprod. Toxicol. 33, 142–154.

Sobanska, M., Scholz, S., Nyman, A.M., Cesnaitis, R., Gutierrez Alonso, S., Kluver, N., Kuhne, R., Tyle, H., de Knecht, J., Dang, Z., Lundbergh, I., Carlon, C., De Coen, W., 2018. Applicability of the fish embryo acute toxicity (FET) test (OECD 236) in the regulatory context of Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH). Environ. Toxicol. Chem. 37, 657–670.

Stelzer, J.A.A., Rosin, C.K., Bauer, L.H., Hartmann, M., Pulgati, F.H., Arenzon, A., 2018. Is fish embryo test (FET) according to OECD 236 sensible enough for delivering quality data for effluent risk assessment? Environ. Toxicol. Chem. 37, 2925–2932.

Stewart, A.G., Carter, J., 2009. Towards the development of a multidisciplinary understanding of the effects of toxic chemical mixtures on health. Environ. Geochem. Health 31, 239–251.

Verhaar, H., Van Leeuwen, C., Hermens, J., 1992. Classifying environmental pollutants. 1: structure-activity relationships for prediction of aquatic toxicity. Chemosphere. 25, 471–491.

Wang, Z., Kwok, K.W., Lui, G.C., Zhou, G.J., Lee, J.S., Lam, M.H., Leung, K.M., 2014. The difference between temperate and tropical saltwater species' acute sensitivity to chemicals is relatively small. Chemosphere. 105, 31–43.

Wang, Z., Scott, W.C., Williams, E.S., Ciarlo, M., DeLeo, P.C., Brooks, B.W., 2018. Identification of novel uncertainty factors and thresholds of toxicological concern for health hazard and risk assessment: application to cleaning product ingredients. Environ. Int. 113, 357–376.

Webster, J., 2005. The assessment and implementation of animal welfare: theory into practice. Rev. Sci. Tech. Off. Int. Epizoot. 24, 723.

Zhang, X., Xia, P., Wang, P., Yang, J., Baird, D.J., 2018. Omics advances in ecotoxicology. Environ. Sci. Technol. 52, 3842–3851.